

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|---|
| (51) International Patent Classification ⁶ : C12Q 1/68 | A1 | (11) International Publication Number: WO 96/32500 (43) International Publication Date: 17 October 1996 (17.10.96) |
| (21) International Application Number: PCT/AU96/00213 (22) International Filing Date: 12 April 1996 (12.04.96) (30) Priority Data: PN 2452 13 April 1995 (13.04.95) AU (71) Applicant (for all designated States except US): JOHNSON & JOHNSON RESEARCH PTY. LIMITED [AU/AU]; 154 Pacific Highway, St. Leonards, NSW 2065 (AU). (72) Inventor; and (75) Inventor/Applicant (for US only): TODD, Alison, Velyian [AU/AU]; 10/66 Campbell Parade, Bondi Beach, NSW 2026 (AU). (74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU). | | (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> |
| (54) Title: METHOD FOR AMPLIFYING SPECIFIC NUCLEIC ACID SEQUENCES | | |
| (57) Abstract <p>The present invention provides a method of detecting a genetic polymorphism in an individual. In one form the method comprising the following steps: (1) obtaining a sample containing nucleic acid from the individual; (2) amplifying the nucleic acid sample from step (1) by a process involving thermocycling and primers, the amplification occurring in the presence of a thermostable restriction endonuclease which retains activity during thermocycling, the primers being selected such that they introduce into either the nucleic acid amplified from nucleic acid not including the polymorphism or from nucleic acid including the polymorphism, a sequence recognised by the thermostable restriction endonuclease; and (3) analyzing the product of step (2) to determine the presence or absence of the polymorphism.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

METHOD FOR AMPLIFYING SPECIFIC NUCLEIC ACID SEQUENCES

FIELD OF INVENTION

5 This invention relates to methods for *in vitro* amplification of specific nucleic acid target sequences. In particular the invention relates to methods which employ thermophilic restriction endonucleases to mediate selective amplification of nucleic acid targets which contain sequence differences including point mutations, deletions and insertions.

BACKGROUND OF THE INVENTION

10 A variety of inherited and acquired diseases are associated with genetic variations such as point mutations, deletions and insertions. Some of these variants are directly associated with the presence of disease, while others correlate with disease risk and/or prognosis. There are of the order of 500 human genetic diseases which result from mutations in single genes. These include cystic fibrosis, muscular dystrophy, α 1-
15 antitrypsin deficiency, phenylketonuria, sickle cell anaemia or trait, and various other haemoglobinopathies. Furthermore, individuals with increased susceptibility to several common polygenic conditions, such as atherosclerotic heart disease, have been shown to have an association with the inheritance of a particular DNA sequence polymorphism. Cancer is thought to develop due the accumulation of lesions in genes
20 involved in cellular proliferation or differentiation. The *ras* proto-oncogenes, *K-ras*, *N-ras*, and *H-ras*, and the p53 tumour suppressor gene are examples of genes which are frequently mutated in human cancers. Specific mutations in these genes leads to activation or increased transforming potential. Genetic analysis is likely to become routine in the clinic for assessing disease risk, diagnosis of disease, predicting a patient's
25 prognosis or response to therapy, and for monitoring a patient's progress. The introduction of such genetic tests depends on the development of simple, inexpensive, and rapid assays for genetic variations.

In rare instances mutations can be detected if they happen to lie within a naturally occurring restriction endonuclease recognition/cleavage site. WO 84/01389
30 describes a method for discriminating between wild type genes and non wild type variants by screening for the presence or absence of restriction endonuclease sites. The inventors demonstrated the principle by analysis of variant sequences at codon 12 of the human *H-ras* proto-oncogene. The wild type sequence at codon 12 forms part of the recognition/cleavage sites for the restriction endonucleases *Nae* I and *Hpa* II.
35 Digestion with these endonucleases can discriminate between the wild type proto-oncogene and activated oncogenes which harbour mutations at this codon. Point

mutations at codon 12 of *H-ras* are frequently found in bladder carcinomas and this general strategy could form the basis of screening kits for medical diagnosis.

Methods of *in vitro* nucleic acid amplification have wide-spread applications in genetics and disease diagnosis. The polymerase chain reaction (PCR) is a powerful, exquisitely sensitive procedure for *in vitro* amplification of specific segments of nucleic acids (R.K. Saiki, et al 1985 Science 230, 1350-1354 and F.F. Chehab, et al 1987 Nature 329, 293-294 and US 4683202 and US 4683195 and US 4800159 and US 4965188 and US 5176995). The PCR is mediated by oligonucleotide primers that flank the target sequence to be synthesized, and which are complementary to sequences that lie on opposite strands of the template DNA. The steps in the reaction occur as a result of temperature cycling (thermocycling). Template DNA is first denatured by heating, the reaction is then cooled to allow the primers to anneal to the target sequence, and then the primers are extended by DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is repeated many times and the products of each round of amplification serve as templates for subsequent rounds. This process results in the exponential amplification of amplicons which incorporate the oligonucleotide primers at their 5' termini and which contain newly synthesized copies of the sequences located between the primers.

The PCR is extremely versatile and many modifications of the basic protocols have been developed. Primers used for the PCR may be perfectly matched to the target sequence or they can contain mismatched and or modified bases. Additional sequences at the 5' end of primers can facilitate capture of PCR amplicons and the inclusion of labelled primers can facilitate detection. The inclusion of mismatched bases within primers can result in the induction of new restriction endonuclease recognition/cleavage sites. These sites can be located completely within the primer sequence. Alternatively, they can span a sequence which lies partially within the primer and partially within the newly synthesized target sequence (J.B. Cohen and A.D. Levinson (1988) Nature 334, 119-124). The general rules for designing primers which contain mismatched bases located near the 3' termini have been established (S. Kwok, et al. (1990) Nucleic Acids Research 18, 999-10005).

Modified primers containing mismatched bases were used to induce novel recognition/cleavage sites for restriction endonucleases in *H-ras* amplicons which were mutated at codon 12 (R. Kumar and M. Barbacid (1988) Oncogene 3, 647-651). Similarly, primers containing mismatched bases were employed in protocols known as allele specific enrichment (Todd AV *et al* Leukemia, 1991; 5:160) or enriched PCR (Levi S *et al* Cancer Res., 1991; 6:1079). These are very sensitive protocols for the

detection of point mutations. In these protocols, DNA samples were amplified with primers which induced either an *Eco* NI site in N-*ras* amplicons, or a *Bst* NI site in K-*ras* amplicons, provided the sequences were wild type at codon 12. Aliquots of the PCR reactions were digested with the appropriate restriction endonuclease to cleave wild type amplicons prior to re-amplification of the digestion-resistant amplicons in a second round of the PCR. These protocols resulted in preferential amplification of sequences harbouring point mutations at codon 12 of *ras*. More recently, a simplified enriched PCR protocol was published which allowed the reaction to be performed in a single tube (Singh *et al* Int J Oncol., 1994; 5: 1009). This protocol also required an initial round of PCR amplification, however, the restriction endonuclease was then added directly to the reaction tube. Following incubation with the restriction endonuclease, a second round of the PCR resulted in amplification of sequences harbouring mutations within the restriction endonuclease recognition/cleavage site. This analysis of natural or induced restriction endonuclease sites in PCR amplicons requires sequential activity of a DNA polymerase for the PCR, followed by activity of a restriction endonuclease for cleavage analysis. Enriched PCR protocols require sequential activity of firstly a DNA polymerase for the PCR, then restriction endonuclease activity to cleave specific sequences, followed by further DNA polymerase activity to re-amplify digestion resistant amplicons.

The ability to simultaneously exploit the activities of a restriction endonuclease and a DNA polymerase during the PCR could provide several advantages. It could allow the development of simple protocols for exclusive or preferential amplification of variant sequences in reactions which contain all reagents, including enzymes, at the initiation of the PCR. It was not previously known whether or not inclusion of a restriction endonuclease in a PCR could result in (i) complete (or partial) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) exclusive (or preferential) amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. The ability to completely inhibit amplification of a sequence and/or exclusively amplify a variant sequence could lead to the development of protocols which do not require further manipulation prior to analysis. A reduction in the number of steps required for selective amplification and/or subsequent analysis of amplicons could lead to the development of protocols which are more rapid, less labour intensive and/or more amenable to automation. A further advantage is that reactions would be performed in a closed system and this would reduce the opportunity for contamination during the PCR

Such protocols would require concurrent activity of a restriction endonuclease and a DNA polymerase under conditions compatible with the PCR. The restriction endonuclease and the DNA polymerase must i) function in identical reaction conditions (eg., salt, pH) which must be compatible with the PCR and ii) must be sufficiently thermostable in these reaction conditions to retain activity during the thermocycling which is required for the PCR. Restriction endonucleases which are suitable for combination with the PCR must be active at temperatures which are compatible with stringent conditions for annealing of primers during the PCR, typically 50°C - 65°C. Simultaneous activity of thermophilic DNA polymerases and restriction endonucleases has previously been exploited to mediate *in vitro* amplification in an isothermal reaction known as strand displacement amplification (EP O 684 315 AI). It was not previously known whether restriction endonucleases could be sufficiently thermostable to maintain activity during the thermocycling required for the PCR.

SUMMARY OF INVENTION

In a first aspect the present invention consists in a method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

- (1) Obtaining a sample containing nucleic acid from the individual;
- (2) Amplifying the nucleic acid sample from step (1) by a process involving thermocycling and primers, the amplification occurring in the presence of a thermostable restriction endonuclease which retains activity during thermocycling, the primers being selected such that they introduce into either the nucleic acid amplified from nucleic acid not including the polymorphism or from nucleic acid including the polymorphism, a sequence recognised by the thermostable restriction endonuclease; and
- (3) Analysing the product of step (2) to determine the presence or absence of the polymorphism.

In one embodiment of this aspect of the present invention the primers introduce the sequence recognised by the thermostable restriction endonuclease into the nucleic acid amplified from the nucleic acid not including the polymorphism.

In a second aspect the present invention consists in a method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

- (1) Obtaining a sample containing nucleic acid from the individual;
- (2) Amplifying the nucleic acid sample from step (1) by a process involving thermocycling and primers, the amplification occurring in the presence of a thermostable restriction endonuclease having concurrent activity, the restriction endonuclease being selected such that it recognises nucleic acid not

including the polymorphism but not nucleic acid including the polymorphism or *vice versa*; and

(3) Analysing the product of step (2) to determine the presence or absence of the polymorphism.

5 In one embodiment of this aspect of the present invention the thermostable restriction endonuclease recognises nucleic acid not including the polymorphism.

In a preferred embodiment of the present invention the method further comprises the following additional steps of:

10 (4) reacting the amplified nucleic acid from step (2) with at least one restriction endonuclease, the at least one restriction endonuclease being selected such that it digests the amplified nucleic acid including a particular polymorphism; and

(5) determining whether digestion occurs in step (4), digestion being indicative of the presence of the particular polymorphism.

15 There are a number of techniques for amplifying nucleic acid which involve thermocycling. These include polymerase chain reaction (PCR), ligase chain reaction, transcription-based amplification and restriction amplification. It is, however, presently preferred that the process involving thermocycling is PCR.

20 In yet a further preferred embodiment the step (3) analysis comprises detecting the presence or absence of amplified nucleic acid from step (2), the presence or absence of amplified nucleic acid indicating the presence or absence of the polymorphism

Whilst the method of the present invention can be used with varying types of nucleic acid typically the nucleic acid will be DNA.

25 In yet another preferred embodiment of the present invention the thermostable restriction endonuclease is selected from the group consisting of *Bst* NI, *Bsl* I, *Trr* 9I and *Tsp* 509 I.

30 The method of the present invention can be used to detect a range of genetic polymorphisms including those occurring in one of the *ras* proto-oncogenes, *K-ras*, *N-ras*, and *H-ras*, or the p53 tumour suppressor gene, or in HIV-1, cystic fibrosis transmembrane conductance regulator, α -antitrypsin or β -globin. The method of the present invention is particularly useful in detecting polymorphisms in codon 12 of *K-ras*.

35 The method of the present invention can be used for the analysis of a range of genetic polymorphisms including point mutations, small deletions and insertions. It was discovered that thermostable restriction endonucleases can be sufficiently thermostable to retain activity during thermocycling. It was also found that the PCR can be performed, using various polymerases, under the same buffer conditions which maintain

activity and thermostability of the restriction endonucleases. It was discovered that the inclusion of a thermostable restriction endonuclease during the PCR can result in (i) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) exclusive amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. These discoveries allowed the development of protocols for restriction endonuclease mediated selective PCR (REMS-PCR). REMS-PCR is simpler than other PCR protocols which utilise restriction endonucleases for the analysis of sequence variations. All components of the reaction are present at the initiation of the PCR and no subsequent manipulations are required prior to analysis. The reaction can therefore be performed in a closed vessel or chamber. It was also found that the inclusion of a thermostable restriction endonuclease during PCR can result in (i) partial inhibition of amplification of nucleic acid which contains the recognition/cleavage site for the restriction endonuclease and (ii) preferential amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the following terms and phases are defined as follows:

The PCR is an *in vitro* DNA amplification procedure which requires two primers that flank the target sequence to be synthesized. A primer is an oligonucleotide sequence which is capable of hybridising in a sequence specific fashion to the target sequence and extending during the PCR. Amplicons or PCR products or PCR fragments are extension products which comprise the primer and the newly synthesized copies of the target sequences. Multiplex PCR systems contain multiple sets of primers which result in simultaneous production of more than one amplicon. Primers may be perfectly matched to the target sequence or they may contain internal mismatched bases which can result in the induction of restriction endonuclease recognition/cleavage sites in specific target sequences. Primers may also contain additional sequences and/or modified or labelled nucleotides to facilitate capture or detection of amplicons. Repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences and extension of the annealed primers with DNA polymerase result in exponential amplification of the target sequence. The terms target or target sequence refer to nucleic acid sequences which are amplified. The term template refers to the original nucleic acid which is to be amplified.

Restriction endonuclease mediated selective PCR (REMS-PCR) is an assay developed by the present inventor which applies the method of the present invention. This assay requires simultaneous activity of a restriction endonuclease and a DNA

polymerase during the PCR. Restriction endonucleases which are suitable for REMS-PCR are preferably active at temperatures which are compatible with stringent conditions for annealing of oligonucleotide primers during the PCR, typically 50°C-65°C. A selection of commercially available restriction endonucleases which have high optimal incubation temperatures in this range are listed below in Table 1.

The term "individual" is used in herein in broadest sense and is intended to cover human and non-human animals, bacteria, yeast, fungi and viruses.

TABLE 1

| Restriction Endonuclease | Recognition/Cleavage Sequence | Optimal Incubation Temperature (s) |
|------------------------------|--------------------------------------|------------------------------------|
| <i>Acc</i> III | TCCGGA | 65°C |
| <i>Acs</i> I/ <i>Apo</i> I | (A/G)AATT(T/C) | 50°C |
| <i>Acy</i> I | G(A/G)CG(C/T)C | 50°C |
| <i>Bco</i> I | C(C/T)CG(A/G)G | 65°C |
| <i>Bsa</i> BI/ <i>Bsi</i> BI | GATNNNNATC | 60°C/55°C |
| <i>Bsa</i> MI | GAATGCN | 65°C |
| <i>Bsa</i> II | CCNNGG | 60°C |
| <i>Bsa</i> OI | CG(A/G)(T/C)CG | 50°C |
| <i>Bsa</i> WI | (A/T)CCGG(A/T) | 60°C |
| <i>Bsc</i> BI | GGNNCC | 55°C |
| <i>Bsc</i> CI | GAATGCN | 65°C |
| <i>Bsc</i> FI | GATC | 55°C |
| <i>Bse</i> AI | TCCGGA | 55°C |
| <i>Bsi</i> CI | TTCGAA | 60°C |
| <i>Bsi</i> EI | CG(A/G)(C/T)CG | 55°C |
| <i>Bsi</i> HKA I | G(A/T)GC(A/T)C | 65°C |
| <i>Bsi</i> LI | CC(A/T)GG | 60°C |
| <i>Bsi</i> MI | TCCGGA | 60°C |
| <i>Bsi</i> QI | TGATCA | 60°C |
| <i>Bsi</i> WI | CGTACG | 55°C |
| <i>Bsi</i> XI | ATCGAT | 65°C |
| <i>Bsi</i> ZI | GGNCC | 60°C |
| <i>Bsl</i> I | CCNNNNNNNGG | 55°C |
| <i>Bsm</i> I | GAATGCN | 65°C |
| <i>Bsm</i> AI | GTCTCN ₁ /N ₅ | 55°C |
| <i>Bsm</i> BI | CGTCTCN ₁ /N ₅ | 55°C |
| <i>Bss</i> TII | CC(A/T)(A/T)GG | 50°C |
| <i>Bsr</i> I | ACTGGN | 65°C |
| <i>Bsr</i> DI | GCAATGNN | 60°C |
| <i>Bst</i> 7II | GCAGCN ₈ | 50°C |
| <i>Bst</i> BI | TTCGAA | 65°C |
| <i>Bst</i> NI | CC(A/T)GG | 60°C |
| <i>Bst</i> UI | CGCG | 60°C |
| <i>Bst</i> YI | (A/G)GATC(C/T) | 60°C |
| <i>Bst</i> ZI | CGGCCG | 50°C |
| <i>Dsa</i> I | CC(A/G)(C/T)GG | 55°C |
| <i>Mae</i> II | ACGT | 55°C |
| <i>Mae</i> III | GTNAC | 55°C |
| <i>Mwo</i> I | GCNNNNNNNGC | 60°C |
| <i>Ssp</i> BI | TGTACA | 50°C |
| <i>Taq</i> I | TCGA | 65°C |
| <i>Tfi</i> I | GA(A/T)TC | 65°C |
| <i>Tru</i> 9I | TTAA | 65°C |
| <i>Tsp</i> 45 I | GT(C/G)AC | 65°C |
| <i>Tsp</i> 509 I | AATT | 65°C |
| <i>Tsp</i> R I | NNCAGTGNN | 65°C |
| <i>Tth</i> 111 I | GACNNNGTC | 65°C |

A=adenine, G=guanine, T=thymine, C=cytosine, and N=A or G or T or C

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described by reference to the following examples.

5

EXAMPLE 1

ASSAY FOR ASSESSING THE ACTIVITY/THERMOSTABILITY OF RESTRICTION ENDONUCLEASES

The activity/thermostability assay was used to examine the thermostability and residual enzymatic activity of restriction endonucleases including *Bst* NI, *Bsl* I, *Tru* 9I, and *Tsp* 509 I, in various buffer systems following a defined number of thermocycles.

The activity/thermostability of *Bst* NI, *Bsl* I and *Tru* 9I was compared for a variety of buffer conditions. Reactions contained primers (as indicated below in Table 2), each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μ M, 0.5 units of *Taq* DNA polymerase (5 units/ μ l; AmpliTaq, Perkin Elmer) and either 20 units of *Bst* NI (10 units/ μ l; New England Biolabs) or *Tru* 9I (10 units/ μ l; Boehringer Mannheim) or 10 units of *Bsl* I (50 units/ μ l; New England Biolabs) in a total reaction volume of 25 μ l.

15

Table 2

| Primer | Amount (pmole) | Present in assay for | Sequence |
|--------|----------------|--|--------------------------|
| 5BKIT | 7.5 | <i>Bst</i> NI | TATAAACTTGTGGTAGTTGGACCT |
| 5BKIQ | 7.5 | <i>Bsl</i> I, <i>Tru</i> 9I | TATAAACTTGTGGTACCTGGAGC |
| 3KiE | 7.5 | <i>Bst</i> NI, <i>Bsl</i> I, <i>Tru</i> 9I | CTCATGAAAATGGTCAGAGAAACC |
| 5BKIW | 1.25 | <i>Bsl</i> I | TTTTGTCGACGAATATGATCC |

20

In addition, reactions contained one of the following basic buffer systems (set out in Table 3) with or without various additional reagents.

Table 3

| Basic Buffer Name *** New England Biolabs ** Boehringer Mannheim * Perkin Elmer | Salt | Tris HCl (pH at 25°C) | MgCl ₂ mM | DTT mM |
|--|--------------------------|---|-------------------------|-----------|
| *** NEB 2 ** SuRE/Cut M | 50 mM NaCl | 10 mM (7.9) | 10 | 1 |
| *** NEB 3 * PCR Buffer II | 100 mM NaCl 50 mM KCl | 50 mM (7.9) 10 mM (8.3) | 10 | 1 |
| * Stoffel Buffer | 10 mM KCl | 10 mM (8.3) | | |
| MTris10 | 50 mM NaCl | 10 mM (8.0 or 8.3 or 8.5 or 8.75) | | |
| Htris50 | 100 mM NaCl | 50 mM (8.0 or 8.3 or 8.5 or 8.75 or 9.0 or 9.5) | | |

The reactions were placed in a GeneAmp PCR system 9600 thermocycler (Perkin Elmer), heated to high temperature and thermocycled as indicated in Table 4.

5

Table 4

| Restriction Endonuclease | <i>Bst</i> NI | <i>Bst</i> I | <i>Tru</i> 9I |
|-----------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| Initial Temperature | 94° C for 2 min. | 92° C for 1 min. | 94° C for 2 min. |
| Thermocycling | 60° C for 1 min 92° C for 20 sec | 55° C for 1 min 92° C for 20 sec | 65° C for 1 min 92° C for 20 sec |
| Number of thermocycles | 15 or 30 | 15 or 30 | 15 or 30 |
| Optimal Incubation Temperature | 60° C | 55° C | 65° C |

Following thermocycling, 8 µg of plasmid DNA in a volume of 5 µl (pGFP-C1; Clontech) was added to each tube and the reactions were incubated for 1 hour at the optimal temperature as indicated by the manufacturer. The ability of the restriction endonuclease to cleave the plasmid DNA was assessed by electrophoresis on 3% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The endonuclease was scored as either being inactivated (I); having low (L), moderate (M) or high (H) activity; or having full (F) activity. (Table 5)

15

Table 5

| Basic Buffer | Additional Reagents | Bst NI Activity | | Bst I Activity | | Tru 91 Activity | |
|---|---|--------------------|----|-------------------|----|--------------------|----|
| | | Cycles | | Cycles | | Cycles | |
| | | 15 | 30 | 15 | 30 | 15 | 30 |
| NEB2 SuRE Cut M | | M | L | | | | |
| NEB3 | | F | M | | | M | L |
| 1 x PCR Buffer II | 3 mM MgCl ₂ | M | L | | | | |
| | 6 mM MgCl ₂ | M | L | I | I | | |
| | 10 mM MgCl ₂ | H | M | I | I | | |
| | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | | |
| 1 x Stoffel Buffer | 3 mM MgCl ₂ | M | L | | | | |
| | 6 mM MgCl ₂ | M | L | I | I | | |
| | 10 mM MgCl ₂ | M | L | I | I | | |
| | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | | |
| MTris 10 pH 8.3 | 10 mM MgCl ₂ | H | L | I | I | | |
| HTris 50 pH 8.3 | | F | M | | | | |
| MTris 10 pH 8.0 | 10 mM MgCl ₂ : 1 mM DTT | | | | | | |
| MTris 10 pH 8.3 | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | | |
| MTris 10 pH 8.0 | 10 mM MgCl ₂ | M | L | | | | |
| MTris 10 pH 8.3 | | H | L | I | I | | |
| MTris 10 pH 8.5 | | M | L | I | I | | |
| MTris 10 pH 8.75 | | | | I | I | | |
| | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | H | L |
| HTris 50 pH 8.5 | 6 mM MgCl ₂ | | | I | I | | |
| HTris 50 pH 8.5 | 6 mM MgCl ₂ : 1 mM DTT | | | H | M | | |
| HTris 50 pH 8.0 | 10 mM MgCl ₂ | F | M | | | | |
| HTris 50 pH 8.3 | | F | M | I | I | | |
| HTris 50 pH 8.5 | | F | M | I | I | | |
| HTris 50 pH 8.5 | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | H | L |
| HTris 50 pH 8.75 | 10 mM MgCl ₂ | | | I | I | | |
| | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | H | L |
| HTris 50 pH 8.3 | 3 mM MgCl ₂ | H | M | | | | |
| | 6 mM MgCl ₂ | F | M | | | | |
| | 10 mM MgCl ₂ | F | M | I | I | | |
| HTris 50 pH 8.3 | 10 mM MgCl ₂ : 1 mM DTT | | | H | M | | |
| HTris 50 pH 8.3 6 mM MgCl ₂ | - | F | M | | | | |
| | 1 mM DTT | H | M | | | | |
| | 0.1 mg/ml acetylated BSA (aBSA) | H | M | | | | |
| | 0.1 mg/ml non-acetylated BSA (non-a BSA) | H | M | | | | |
| | 1 mM DTT + aBSA | M | M | | | | |
| | 1 mM DTT + non-aBSA | M | L | | | | |
| | 10 % glycerol | H | M | | | | |
| HTris50 pH 9.0 | 10 mM MgCl ₂ : 1 mM DTT | | | | | H | M |
| HTris50 pH 9.5 | 10 mM MgCl ₂ : 1 mM DTT | | | | | F | M |

These experiments indicated that the activity/thermostability of restriction endonucleases during thermocycling varied considerably depending on both the restriction endonuclease and buffer system in which it was assayed. The pH and ionic strength of the Tris buffer, the choice and concentration of monovalent cation (K^+ or Na^+), the concentration of free Mg^{2+} , and the presence of other additives, particularly DTT, could influence the activity/thermostability. The influence of each of these components could depend on the other components in the buffer. For example, *Bst* NI retained more activity in PCR Buffer II containing 10 mM $MgCl_2$ than in this buffer containing either 3 or 6 mM $MgCl_2$. In contrast, varying the concentration of $MgCl_2$ between 3 and 10 mM had little effect on *Bst* NI activity when in HTris 50 (pH 8.3) buffer. In another example, the pH of the buffer had a greater influence on thermostability/activity of *Bst* NI in MTris 10 than in HTris 50.

Bst NI remains fully active following 15 thermocycles and moderately active following 30 thermocycles in buffer systems which contain either i) 100 mM NaCl, 50 mM Tris HCl (pH 8.3) and 6 mM $MgCl_2$ or ii) 100 mM NaCl, 50 mM Tris HCl (pH 8.0 - 8.5) and 10 mM $MgCl_2$ or iii) NEB 3 buffer and 0.1 mg/ml. *Bst* NI is more active in these buffers during thermocycling than in NEB 2 buffer with 0.1 mg/ml acetylated BSA which are the buffer conditions recommended by the manufacturers.

Similar experiments examining activity/thermostability of *Bst* I indicated that this endonuclease requires the presence of 1 mM DTT in order to remain active following thermocycling. Provided DTT is present, *Bst* I remains active in a broad range of conditions. *Bst* I retains moderate activity following 30 thermocycles in buffer systems which contain i) PCR buffer II (Perkin Elmer), 1 mM DTT and 10 mM $MgCl_2$ ii) Stoffel buffer (Perkin Elmer), 1 mM DTT and 10 mM $MgCl_2$ iii) 50 mM NaCl, 10 mM Tris HCl (pH 8.5), 1 mM DTT and 10 mM $MgCl_2$ iv) 100 mM NaCl, 50 mM Tris HCl (pH 8.3 - 8.5), 1 mM DTT and 10 mM $MgCl_2$ or v) 100 mM NaCl, 50 mM Tris HCl (pH 8.5), 1 mM DTT and 6 mM $MgCl_2$. *Tru* 9I retains moderate activity following 30 thermocycles in a buffer system which contains 100 mM NaCl, 50 mM Tris HCl (pH 8.5 - 9.25), 10 mM $MgCl_2$ and 1 mM DTT. Experiments similar to those described above showed that *Tsp* 509 I retains moderate activity following 30 thermocycles in a buffer system which contains 50 mM NaCl, 10 mM Tris HCl (pH 9.0 to 10), 10 mM $MgCl_2$ and 1mM DTT.

EXAMPLE 2**IDENTIFICATION OF BUFFER SYSTEMS COMPATIBLE WITH
RESTRICTION ENDONUCLEASE AND DNA POLYMERASE
THERMOSTABILITY/ACTIVITY AND THE PCR.**

5 The range of buffers which was assessed for ability to maintain
thermostable/activity of *Bst* NI (above) was also assessed for compatibility with the
PCR using primers 5BKIT or 5BKIW with 3KiE. The PCR mixtures containing
genomic K562 DNA (800 ng), 30 pmole of 5BKIT or 30 pmole 5BKIW, 30 pmole of
3KiE, and each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μ M were set up for various
10 buffer systems. Four units of *Taq* DNA polymerase (5 units/ μ l; AmpliTaq, Perkin
Elmer) were mixed with TaqStartTM antibody (0.16 μ l in 3.8 μ l of antibody dilution
buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStartTM
antibody of 1:5. The *Taq* DNA polymerase:TaqStartTM antibody mixture was
incubated for 15 min at room temperature prior to addition to the mixtures. The total
15 reaction volumes were 100 μ l. The reactions were placed in a GeneAmp PCR system
9600 (Perkin Elmer), denatured at 94 $^{\circ}$ C for 2 min and then subjected to 30 cycles of
60 $^{\circ}$ C for 1 min followed by 92 $^{\circ}$ C for 20 sec. Reactions were held at 60 $^{\circ}$ C for 15
min after thermocycling.

 A 28 μ l aliquot of each reaction was analysed without subsequent
20 manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts,
Rockland, MD). The gel was photographed using Stratagene Eagle Eye II video
system. The efficiency of amplification with primers 5BKIT and 3KiE, or 5BKIW and
3KiE was rated as low, moderate or high. These primers were designed for use in a
multiplex REMS-PCR system in conjunction with the restriction endonuclease *Bst* NI.
25 The activity/thermostability assay on *Bst* NI and the PCR were performed in the same
reaction buffers and subjected to the same thermocycling profile. The results of the
two assay were examined to find conditions which allowed both efficient PCR
amplification and preservation of restriction endonuclease activity. (Table 6)

Table 6

| Basic Buffer | Additional Reagents | PCR Amplification Efficiency | | <i>Bst</i> NI Activity | |
|---|-------------------------|------------------------------|---------------|------------------------|-----------|
| | | 5BKIT 3KiE | 5BKIW 3KiE | 15 cycles | 30 cycles |
| NEB2 | | High | High | Moderate | Low |
| NEB3 | | High | Moderate | Full | Moderate |
| PCR Buffer II | 3 mM MgCl ₂ | High | High | Moderate | Low |
| | 6 mM MgCl ₂ | High | High | Moderate | Low |
| | 10 mM MgCl ₂ | High | High | High | Moderate |
| Stoffel Buffer | 3 mM MgCl ₂ | High | High | Moderate | Low |
| | 6 mM MgCl ₂ | High | High | Moderate | Low |
| | 10 mM MgCl ₂ | Moderate | Moderate | Moderate | Low |
| MTris 10 pH 8.3 | 10 mM MgCl ₂ | High | High | High | Low |
| HTris 50 pH 8.3 | | Moderate | Moderate | Full | Moderate |
| MTris 10 pH 8.0 | 10 mM MgCl ₂ | High | Moderate | Moderate | Low |
| MTris 10 pH 8.3 | | High | High | High | Low |
| MTris 10 pH 8.5 | | High | Moderate | Moderate | Low |
| HTris 50 pH 8.0 | 10 mM MgCl ₂ | Moderate | Moderate | Full | Moderate |
| HTris 50 pH 8.3 | | Moderate | Moderate | Full | Moderate |
| HTris 50 pH 8.5 | | Low | Moderate | Full | Moderate |
| HTris 50 pH 8.3 | 3 mM MgCl ₂ | High | Moderate | High | Moderate |
| | 6 mM MgCl ₂ | High | Moderate | Full | Moderate |
| | 10 mM MgCl ₂ | Moderate | Moderate | Full | Moderate |
| HTris 50 pH 8.3 6 mM MgCl ₂ | - | High | Moderate | Full | Moderate |
| | DTT | High | Moderate | High | Moderate |
| | aBSA | High | Moderate | High | Moderate |
| | non-a BSA | High | Moderate | High | Moderate |
| | DTT + a BSA | High | Moderate | Moderate | Moderate |
| | DTT + non-a BSA | High | Moderate | Moderate | Low |
| | glycerol | Low | Low | High | Moderate |
| | T4 gene 32 protein | High | Low | Low | Inactive |

- The buffer conditions which simultaneously i) resulted in highly efficient amplification with the primer pair 5BKIT and 3KiE and moderately efficient amplification of the primer pair 5BKIW and 3KiE and ii) preserved full *Bst* NI activity for at least 15 thermocycles and moderate activity for 30 thermocycles, were selected for use in a REMS-PCR assay which requires concurrent activity of DNA *Taq* polymerase and *Bst* NI. Buffer conditions that fit these criteria were 100 mM NaCl, 50 mM Tris HCl pH 8.3 and 6 mM MgCl₂.

EXAMPLE 3**REMS-PCR USING *Bst*NI and DNA *Taq* POLYMERASE: ANALYSIS OF CODON 12 OF THE K-RAS GENE IN A MULTIPLEX SYSTEM INCORPORATING INTERNAL CONTROLS.**

- 5 A REMS-PCR protocol was used to detect point mutations at codon 12 of the *K-ras* oncogene. The human cell lines Calu I [ATCC HTB54] and K562 [ATCC CCL243] were obtained from the American Type Culture Collection. Calu I is a lung adenocarcinoma cell which is heterozygous at *K-ras* codon 12 having both wild type (GGT) and mutant (TGT) sequences (D.J. Capon 1983 Nature 304, 507-513).
- 10 K562 is a human leukemic cell line which is wild type at codon 12 of *K-ras* (R.L. Ward et al. Mol Pathol 1995 48, M273-277). Genomic DNA was extracted from Calu I and K562 by standard techniques (Sambrook et al 1989). DNA samples were amplified by REMS-PCR using primers 5BKIT, 5BKIW, 3MKiC and 3KiE. (Table 7)

15 Table 7

| Primer | Function | Sequence |
|--------|------------------------------|-----------------------------|
| 5BKIT | Diagnostic primer | TATAAACTTGTGGTAGTTGGACCT |
| 5BKIW | PCR control primer | TTTGTGCGACGAATATGATCC |
| 3MKiC | <i>Bst</i> NI control primer | CTGTATCAAAGCTTGGTCCTGGACCAG |
| 3KiE | 3' primer | CTCATGAAAATGGTCAGAGAAAC |

- The bold type C in the primer 5BKIT is mismatched with respect to the sequence of the *K-ras* gene. This mismatched base results in the induction of the recognition/cleavage site for *Bst* NI in *K-ras* amplicons provided that they are wild type
- 20 at codon 12. Amplicons containing a mutation at either the first or second nucleotide of codon 12 do not contain the recognition/cleavage sequence for *Bst* NI. Primers 5BKIT and 5BKIW were biotin-labelled at their 5' ends and generate PCR amplicons which are similarly labelled. The bold type G in the primer 3MKiC is mismatched with the *K-ras* sequence. This results in the induction of a *Bst* NI recognition/cleavage site
- 25 which is internal to the primer and which would be incorporated into any amplicons generated by amplification with this primer and either 5BKIT or 5BKIW.

- Genomic DNA from K562, Calu I, and a 1:10 mixture (by weight) of Calu I:K562, was amplified in a multiplex REMS-PCR system. The reactions contained genomic DNA (800 ng), 30 pmole of 5BKIT, 30 pmole of 3KiE, 5 pmole of
- 30 5BKIW, 80 pmole of 3MKiC, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μ M, 80 units of *Bst* NI (10 units/ μ l, New England Biolabs) and 4 units of *Taq* DNA polymerase (5 units/ μ l, AmpliTaq, Perkin Elmer) in 100 mM NaCl, 50 mM Tris (pH 8.3) and 6

mM MgCl₂. The total reaction volumes were 100 µl. Two control reactions contained either Calu I DNA or dH₂O (no DNA) in the absence of *Bst*NI. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec.

- 5 Reactions were held at 60° C for 15 min following thermocycling.

A 25 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. In the control reaction, containing Calu I DNA in the absence of *Bst* NI, three fragments were clearly visible; a 185 bp fragment comprised of amplicons incorporating primers 5BKIT and 3KiE, a 156 bp fragment comprised of amplicons incorporating primers 5BKIT and 3MKiC, and a 114 bp fragment comprised of amplicons incorporating primers 5BKIW and 3KiE. A fragment of 85 bp comprised of amplicons incorporating primers 5BKIW and 3MKiC was faintly visible.

- 15 In the reactions containing *Bst* NI, the presence of the 185 bp fragment was diagnostic for the presence of K-*ras* codon 12 mutations. This fragment was visible in reactions containing Calu I and Calu I:K562 DNA at a ratio of 1:10, but not in reactions containing K562 DNA alone. The 156 bp (and 85 bp) *Bst* NI control fragments were not visible in any reactions containing *Bst* NI. This demonstrates that
- 20 *Bst* NI can mediate complete inhibition of amplification of a second fragment. Since any 156 bp amplicon would contain a *Bst* NI site, inhibition of amplification of this fragment is not dependent on the mutational status of codon 12. Absence of restriction endonuclease control fragments allows unambiguous interpretation of negative results. The 114 bp PCR control fragment was visible in all reactions including the reaction
- 25 containing K562 DNA. This confirms that the conditions of the reactions, including the amount of template DNA, were adequate for amplification by the PCR. The presence of PCR control fragments allows unambiguous interpretation of positive results. No fragments were visible in the reaction containing no template DNA.

30 **EXAMPLE 4**

REMS-PCR: LIMIT OF DETECTION OF POINT MUTATIONS

- The limits of detection of point mutations using REMS-PCR were assessed by analysing codon 12 of the K-*ras* gene in samples containing Calu I DNA diluted with Sup T1 DNA. Sup T1 [ATCC CRL 1942] is a leukemia cell line which was obtained
- 35 from the American Type Culture Collection. Calu I is heterozygous mutant at K-*ras* codon 12 and Sup T1 is wild type at codon 12 of the K-*ras* gene. Genomic DNA was

extracted from these cell lines by standard techniques (Sambrook et al 1989) and amplified by the REMS-PCR. Calu I DNA was diluted with Sup T1 DNA at a ratio (by weight) of Calu I:Sup T1 of 1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵ and 1:10⁶.

The REMS-PCR reactions contained genomic DNA (1 µg), 30 pmole of 5BK1T, 30 pmole of 3KiE, 5 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, and 40 units of *Bst* NI (10 units/µl, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.3) and 6 mM MgCl₂. Four units of *Taq* DNA polymerase (5 units/µl; AmpliTaq, Perkin Elmer) were mixed with TaqStartTM antibody (0.16 µl in 3.8 µl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStartTM antibody of 1:5. The *Taq* DNA polymerase:TaqStartTM antibody mixture was incubated for 15 min at room temperature prior to addition to the PCR mixture. The total reaction volumes were 100 µl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 2 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

A 28 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera and the Stratagene Eagle Eye II video system. The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu I:Sup T1 DNA at ratios of 1:10, 1:10² and 1:10³ by polaroid photography and Eagle Eye imaging and in the reaction containing a ratio of 1:10⁴ by Eagle Eye imaging. This 185 bp fragment was not visible in the reactions containing Calu I:Sup T1 DNA at ratios of 1:10⁵ and 1:10⁶ nor in the reaction containing Sup T1 only. The 114 bp PCR control fragment generated by amplification with primers 5BKIW and 3KiE fragment was visible in all reactions. This confirms reaction conditions, including the amount of template DNA, were adequate for efficient amplification by the PCR.

The REMS-PCR reactions were also analysed in a colorimetric assay. This assay is similar to that described in Findlay et al (Clin. Chem. 1993 39/9, 1927-1933). PCR amplicons were specifically captured by hybridization to oligonucleotide probes that were covalently attached to latex beads which were applied at discrete locations in Periodontal Surecell blanks. The sequence of the capture oligonucleotides, and the specific PCR amplicons captured, are listed below (Table 8). K-Cap1 and K-Cap 2 were specifically designed to capture only diagnostic K-ras amplicons which were

generated by amplification of mutant templates with the primers 5BKIT and 3KiE. K-Cap 3 is designed to capture amplicons generated by amplification of either mutant and wild type templates with either 5BKIT or 5BKIW and 3KiE. H-Cap 1 captures non-specific amplicons and provides a negative control for non-specific amplification or hybridization.

Table 8

| Probe (Function) | Sequence | Sizes of fragments with homology (Primers incorporated) | Type(s) of amplicons captured |
|------------------------------|----------------------------------|---|-------------------------------------|
| K-Cap 1 (Diagnostic) | TAGCTGTATCGTCAAGGCA CTCTT | 185 bp (5BKIT/3KiE) | Mutant only |
| K-Cap 2 (Diagnostic) | AAATGATTCTGAATTAGCT GTATCGTC | 185 bp (5BKIT/3KiE) | Mutant only |
| K-Cap 3 (PCR control) | GCACCAGTAATATGCATAT TAAAACAAG | 185 bp (5BKIT/3KiE) 114 bp (5BKIW/3KiE) | Mutant Wild type |
| H-Cap1 (Negative control) | ACCATCCAGCTGATCCAGA ACCAT | Nil | Non- specific |

Aliquots of the four oligonucleotide latex beads (0.25% in 1.6 µl of 10mM Tris 1mM EDTA pH 7.4) were applied on to the Surecell membrane in discrete spots with all four oligonucleotides in each Surecell well. The oligonucleotide latex beads were allowed to dry for 15 minutes. Aliquots of 30 µl of each PCR was diluted with 170 µl of 50 mM KCl, 10 mM Tris (pH8.3) and 10 mM MgCl₂. The solution was denatured at 95° C for 6 min and applied to the Surecell well. The Surecells were then incubated at 50° C for 5 min to allow hybridization of PCR amplicons with the capture oligonucleotides. The wells were washed with 300 µl of 50 mM KCl, 10 mM Tris (pH 8.3) and 10 mM MgCl₂ at 50° C. The hybridized amplicons were reacted with three drops of a conjugate of streptavidin bound to horseradish peroxidase (EC 1.11.1.7) and incubated at room temperature for 2 min. The wash step was repeated to minimize non-specific interactions. Four drops of Leucodye/H₂O₂ were added and the Surecell were incubated at room temperature for 2 min. The immobilized complex served as a catalyst in the oxidative conversion of dye molecules from colourless to blue form. The reaction was stopped with 4 drops of 0.1% NaN₃. The resultant coloured spots were scored visually by comparison against a colour chart and rated from 0 (no colour) to 10 (dark blue) (Table 9)

Table 9

| Colour Score | | | | | | | |
|---|------|-------------------|-------------------|-------------------|-------------------|-------------------|--------|
| Calu I:Sup T1 DNA | 1:10 | 1:10 ² | 1:10 ³ | 1:10 ⁴ | 1:10 ⁵ | 1:10 ⁶ | Sup T1 |
| K- Cap 1 (Mutant specific) | 9 | 8 | 4 | 2 | 0 | 0 | 0 |
| K- Cap 2 (Mutant specific) | 9 | 8 | 4 | 2 | 0 | 0 | 0 |
| K- Cap 3 (PCR Control) | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| H - Cap 1 (Non-specific negative control) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

5 The sensitivity of the REMS-PCR protocol allowed detection of selectively amplified mutant sequences of *K-ras* codon 12 when present in a background of 1:10³ to 1:10⁴ wild type sequences when analysed by gel electrophoresis or a colorimetric assay. Wild type *K-ras* codon 12 sequences were not detected in this REMS-PCR assay. The literature suggests that this level of sensitivity will be adequate for analysis of DNA extracted from clinical specimens including tissue resections and biopsies, cytology samples and body fluids/excretions such as stools, urine and sputum containing small numbers of exfoliate tumour cells.

10 In a clinical setting, where large numbers of samples are simultaneously analysed, it is desirable that amplification does not commence prematurely as this can cause amplification of non-specific products including primer dimers. Monoclonal antibodies can bind to DNA *Taq* polymerase, and thus inhibit activity and amplification prior to the first denaturation step. In initial experiments using REMS-PCR, the standard molar ratio of DNA *Taq* polymerase:TaqStartTM of 1:28, as recommended by Clontech, resulted in false positive results due to amplification of wild type Sup T1 DNA templates. Various molar ratios were tested and it was established that lower molar ratios of DNA *Taq* polymerase:TaqStartTM antibody such as 1:5 resulted in inhibition of non-specific amplification and primer dimer formation in the absence of false positive results.

EXAMPLE 5

ANALYSIS OF CLINICAL SPECIMENS USING REMS-PCR.

25 Genomic DNA was extracted by standard protocols (Sambrook et al 1989) from normal colon mucosa (NC) and colon adenocarcinomas (CA). Samples were analysed for the presence of *K-ras* codon 12 mutations by REMS-PCR as outlined in

example 4 with the following protocol changes; DNA (0.5 µg) was amplified in the presence of 4 units of *Taq* DNA polymerase and 80 units of *Bst* NI. A 30 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD).

5 The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible by gel electrophoresis in two reactions containing DNA from adenocarcinoma samples CA7 and CA8. This diagnostic fragment was not visible in two other reactions containing DNA from adenocarcinoma samples CA1 and CA2, nor in four reactions
10 containing DNA extracted from normal colon mucosa; NC1, NC2, NC7 and NC8. The 114 bp control fragment generated by amplification with primers 5BKIW and 3KiE was visible in all reactions, indicating efficient PCR amplification had occurred in all reactions.

 Genomic DNA from colon tissues had previously been analysed for the
15 presence of mutations at *K-ras* codon 12 by standard enriched PCR (R.L. Ward et al Mol Pathol 1995 48, M273-277). Identical results were obtained when amplification was performed by either REMS-PCR or enriched PCR followed by analysis by gel electrophoresis. Both protocols indicated that DNA from adenocarcinoma samples CA7 and CA8 harboured mutations at *K-ras* codon 12 whereas the DNA from
20 adenocarcinomas CA1 and CA2, as well as normal mucosa samples NC1, NC2, NC3 and NC4, were wild type at codon 12. These results demonstrate that REMS-PCR is suitable for rapid analysis of clinical specimens.

25 **EXAMPLE 6: REMS-PCR: A SYSTEM WHICH ALLOWS IDENTIFICATION OF THE SPECIFIC NUCLEOTIDE SUBSTITUTION.**

 A REMS-PCR system was used to detect point mutations at codon 12 of the *K-ras* oncogene. Additional analysis with restriction endonucleases both confirmed the diagnosis of a mutation at codon 12 and allowed identification of the specific nucleotide substitution. The human cell lines Calu I [ATCC HTB54], A549 [ATCC],
30 K562 [ATCC CCL243], Sup T1 [ATCC CRL 1942] and were obtained from the American Type Culture Collection. Calu I is a lung adenocarcinoma cell which is heterozygous at *K-ras* codon 12 having both wild type (GGT) and mutant (TGT) sequences (D.J. Capon 1983 Nature 304, 507-513). A549 is lung adenocarcinoma cell which is homozygous mutant (AGT) at *K-ras* codon 12 (D.M. Valenzuela and J.
35 Groffen 1986 NAR 14, 843-852). K562 and Sup T1 are leukemic cell lines which are

wild type at codon 12 of *K-ras*. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989).

REMS-PCR was performed with primers 5BKIT and 3AKIP which simultaneously induce multiple restriction endonuclease recognition/cleavage sites.

- 5 Primers 5BK5 and 3K6 function as PCR control primers. (Table 10)

Table 10

| Primer | Sequence : Bases mismatched with the <i>K-ras</i> gene which result in induction of restriction sites are indicated in bold type. (Additional mismatched bases are underlined) |
|--------|---|
| 5BKIT | TATAAACTTGTGGTAGTTGGACCT |
| 3AKIP | GGATGACTCATTAAAGGCACTCTTGCCTACGCCC |
| 5BK5 | TCAGCAAAGACAAGACAGGTA |
| 3K6 | AGCAATGCCCTCTCAAGA |

- 10 The primer 5BKIT results in induction of a *Bst* NI recognition/cleavage site in *K-ras* amplicons which are wild type at codon 12. The primer 3AKIP induces one or more recognition/cleavage site(s) for the group of restriction endonucleases *Bsa* II, *Sty* I, *Avr* II, *Mnl* I, *Aci* I, *Rle* I and *Bsu* 36I, in *K-ras* amplicons which are mutated at codon 12 as indicated below (Table 11).

15 Table 11

| K-ras sequences and induced restriction endonuclease recognition/cleavage sites. (Mismatched bases introduced by 5BKIT (C) and 3AKIP (G) are indicated in bold type: Point mutations at codon 12 are underlined: N = T or A or C or G) | | | | |
|---|------------|-------------------|----------|--------------------------------|
| | Codon 11 | Codon 12 | Codon 13 | Restriction Endonuclease(s) |
| Wild Type sequence | CCT CCT | GGG GG | GGC | <i>Bst</i> NI |
| Mutant sequences | CCN | <u>NGG</u> | | <i>Bsa</i> II |
| | CCT | <u>TGG</u> | | <i>Sty</i> I |
| | CCT | <u>AGG</u> | | <i>Avr</i> II/ <i>Sty</i> I |
| | CCT | <u>C</u> | | <i>Mnl</i> I |
| | | GCG | G | <i>Aci</i> I |
| | T | GTG | GG | <i>Rle</i> AI |
| | CCT | <u>NAG</u> GAG | G G | <i>Bsu</i> 36I <i>Mnl</i> I |

The expected pattern of sensitivity and resistance of mutant amplicons to cleavage with the group of restriction endonucleases *Bsa* II, *Sty* I, *Avr* II, *Mnl* I, *Aci* I, *Rle* I and *Bsu* 36I depends upon the exact mutation present at codon 12 and is indicated in Table 12.

5

Table 12

| Codon 12 positions 1 and 2 (Point mutations are underlined; N = T or A or C) | Restriction endonucleases which cleave mutant amplicons | Restriction endonucleases which do not cleave mutant amplicons |
|--|---|--|
| <u>NG</u> | <i>Bsa</i> II | |
| <u>TG</u> | <i>Bsa</i> II/ <i>Sty</i> I | <i>Avr</i> II |
| <u>AG</u> | <i>Bsa</i> II/ <i>Sty</i> I/ <i>Avr</i> II | |
| <u>CG</u> | <i>Bsa</i> II/ <i>Mnl</i> I | <i>Bsu</i> 36I |
| <u>GN</u> | | <i>Bsa</i> II |
| <u>GT</u> | <i>Rle</i> AI | <i>Bsa</i> II |
| <u>GC</u> | <i>Aci</i> I | <i>Bsa</i> II |
| <u>GA</u> | <i>Mnl</i> I/ <i>Bsu</i> 36I | <i>Bsa</i> II |

Genomic DNA from the human cell lines Calu I, A549, K562 and Sup T1 was amplified in a multiplex REMS-PCR system. The reactions contained genomic DNA (500 ng), 50 pmole of 5BK1T, 50 pmole of 3AK1P, 3 pmole of 5BK5, 3 pmole of 3K6, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, 40 units of *Bst* NI (10 units/μl, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.3) and 6 mM MgCl₂. Four units of *Taq* DNA polymerase (5 units/μl; AmpliTaq, Perkin Elmer) were mixed with TaqStartTM antibody (0.06 μl in 1.5 μl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStartTM antibody of 1:2. The *Taq* DNA polymerase:TaqStartTM antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 100 μl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

A 20 μl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. A 58 bp fragment, generated by amplification with primers 5BK1T and 3AK1P, was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu I and A549 DNA but was not visible in reactions containing Sup T1 or K562 DNA. A 167 bp PCR control fragment, generated by amplification with primers

5BK5 and 3K6 was present in all reactions, including reactions containing Sup T1 and K562 DNA. This confirmed that efficient PCR amplification had occurred in all reactions.

5 A 15 µl aliquot of the reactions containing Calu I or A549 DNA was digested with 10 units of the restriction endonucleases from the group *Bsa* II, *Sty* I, *Avr* II, *Mnl* I, *Aci* I (as indicated below in Table 13) and incubated at the optimum temperature for digestion as specified by the manufacturer (New England Biolabs). The reactions were analysed by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD) and the gel was photographed using a polaroid land camera.

10

Table 13

| Template DNA | Primers which generated amplicons | Restriction Endonuclease | Result | Sequence at codon 12 positions 1 and 2 (N = A, C or T) |
|--------------|-----------------------------------|--|---------------------------------|---|
| K562 | 5K5/3K6 only | - | - | Wild type - GG |
| Sup T1 | 5K5/3K6 only | - | - | Wild type - GG |
| Calu I | 5BKIT/3AKIP5K 5/3K6 | <i>Bsa</i> II <i>Sty</i> I <i>Avr</i> II | cleaves cleaves resistant | Mutant NG TG or AG not AG Result: Mutant (TG) |
| A549 | 5BKIT/3AKIP 5K5/3K6 | <i>Bsa</i> II <i>Sty</i> I <i>Avr</i> II | cleaves cleaves cleaves | Mutant NG AG or TG AG Result: Mutant (AG) |

15 This REMS-PCR system allows detection of mutations at codon 12 of the *K-ras* oncogene. Subsequent analysis by restriction endonucleases confirms the presence of the mutation and allows identification of the specific nucleotide substitution.

EXAMPLE 7: REMS-PCR SYSTEM USING *Bst* NI AND STOFFEL POLYMERASE.

20 Genomic DNA from the human cell lines Calu I [ATCC HTB54] and Sup T1 [ATCC CRL 1942] was amplified by the REMS-PCR. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989). DNA was amplified by REMS-PCR in reactions containing genomic DNA (1 µg), 30 pmole of 5BK1T, 30 pmole of 3KiE, 2 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, and 40 units of *Bst* NI (10 units/µl, New England Biolabs) in 10 mM KCl, 10 mM Tris (pH 8.3) and 10 mM MgCl₂ (1 × Stoffel buffer; Perkin Elmer)

25

A control reaction contained no DNA (dH₂O). Five units of Stoffel fragment (10 units/μl; Perkin Elmer) were mixed with Taq antibody TP4 (D.J. Sharkey et al 1994 Bio/technology 12, 506-509) (0.05 μl in 1.2 μl of Clontech antibody dilution buffer) to give a final molar ratio of Stoffel fragment: Taq antibody TP4 of 1:2. The Stoffel fragment: Taq antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 100 μl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 2 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

A 25 μl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible in the reaction containing Calu I DNA, but was not visible in the reaction containing Sup T1 DNA. The 114 bp PCR control fragment, generated by amplification with primers 5BKIW and 3KiE, was visible in both reactions indicating efficient amplification by the PCR. No fragments were visible in the control reaction containing no template.

20 **EXAMPLE 8: REMS-PCR SYSTEM USING *Bst* I AND Taq DNA POLYMERASE.**

A REMS-PCR assay was developed to detect point mutations at codon 12 of the K-ras oncogene. In this assay, amplicons contain the recognition/cleavage sequence for the thermophilic restriction endonuclease *Bst* I provided they are wild type at codon 12. Amplicons which contain a mutation at either the first or second nucleotide of codon 12 do not contain the recognition/cleavage sequence for *Bst* I.

Genomic DNA from the human cell lines Calu I [ATCC HTB54] and K562 [ATC CCL243] was amplified by the REMS-PCR. Calu I is heterozygous mutant at codon 12 of the K-ras gene and K562 is wild type at codon 12. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989). Calu I DNA was diluted with K562 DNA at a ratio (by weight) of Calu I:K562 of 1:10, 1:10² and 1:10³.

DNA was amplified by REMS-PCR using primers 5BKIQ, 5BKIW and 3KiH (Table 14). The 2 bold type C's in 5BKIQ are mismatched with respect to the sequence of the *K-ras* gene. These mismatched bases cause the induction of a *Bs*/I site in amplicons which are wild type at codon 12. Primers 5BKIQ and 5BKIW are
 5 biotinylated.

Table 14

| Primer | Sequence |
|--------|-------------------------|
| 5BKIQ | TATAAACTTGTGGTACCTGGAGC |
| 5BKIW | TTTGTGCGACGAATATGATCC |
| 3KiH | GAAAATGGTCAGAGAAACC |

The reactions contained genomic DNA (400 ng), 30 pmole of 5BKIQ, 15
 10 pmole of 3KiH, 0.5 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μ M, and 10 units of *Bs*/I (50 units/ μ l, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.5), 1 mM DTT and 6 mM MgCl₂. Eight units of *Taq* DNA polymerase (5 units/ μ l; AmpliTaq, Perkin Elmer) were mixed with TaqStartTM antibody (0.16 μ l in 3.8 μ l of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA
 15 polymerase:TaqStartTM antibody of 1:5. The *Taq* DNA polymerase:TaqStartTM antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 50 μ l. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer) and denatured at 94° C for 2 min. The reactions were then subjected to 10 cycles of 63° C for 30 sec followed by 92° C for
 20 20 sec and then 20 cycles of 55° C for 1 min followed by 92° C for 20 sec. Reactions were held at 55° C for 15 min following thermocycling.

A 28 μ l aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. The 180 bp
 25 fragment generated by amplification with primers 5BKIQ and 3KiH was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu I:K562 at a ratio of 1:10 and 1:10². This 180 bp diagnostic fragment was not visible in the reactions containing Calu I:Sup T1 at a ratio 1:10³ or in the reactions containing K562 only. The 109 bp PCR control fragment, generated by
 30 amplification with primers 5BKIW and 3KiH, was visible in all reactions indicating efficient amplification by the PCR.

This system utilized the restriction endonuclease *Bs*/I for detection of mutations at *K-ras* codon 12. This restriction endonuclease could be used in systems

for the detection of mutations that occur at either codons 12 or 13 of any of the three *ras* oncogenes, *K-ras*, *H-ras* and *N-ras*. It could also be used for analysis of other mutations that occur in codons encoding either glycine or proline and for other mutations that occur at the nucleotides C or G.

5

**EXAMPLE 9: ANALYSIS OF K-*ras* CODON 12 BY A REMS-PCR
PROTOCOL WHICH REQUIRES SUBSEQUENT DIGESTION WITH *Bst* NI.**

An alternative protocol was used to detect point mutations at codon 12 of the *K-ras* oncogene. Genomic DNA was extracted from Calu I [ATCC HTB54] and K562
10 [ATCC CCL243] by standard techniques (Sambrook et al 1989). Calu I DNA was diluted with K562 DNA at a ratio (by weight) of Calu I:K562 of 1:10, 1:10², 1:10³ and 1:10⁴. DNA samples were amplified using primers 5BKIM which has the sequence GACTGAATATAAACTTGTGGTAGTTGGACCT and 3AKIL which has the sequence GGATGACTCATTTTTCGTCCACAAAATGATTCTGAATTAG. The bold
15 type C in the primer 5BKIM is mismatched with respect to the sequence of the *K-ras* gene and results in the induction of the recognition/cleavage site for *Bst* NI in *K-ras* amplicons provided that they are wild type at codon 12. Bases within 3AKIL which are mismatched with *K-ras* are underlined.

The reactions contained genomic DNA (800 ng), 40 pmole of 5BKIM and 40
20 pmole of 3AKIL, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 µM, 10 µl of 10 X PCR Buffer II (Perkin Elmer), 1.5 mM MgCl₂, 80 units of *Bst* NI (10 units/µl, New England Biolabs) and 2 units of *Taq* DNA polymerase (5 units/µl; AmpliTaq, Perkin Elmer) in a total reaction volume of 100 µl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then
25 subjected to 40 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min following thermocycling.

A 25 µl aliquot of each reaction was analysed without subsequent manipulation. A second 25 µl aliquot of each reaction was incubated with 15 units of *Bst* NI (10 units/µl, New England Biolabs), 100 µg/ml bovine serum albumin (New
30 England Biolabs) and 3.5 µl of 10 X NEB2 buffer (New England Biolabs) in a total reaction volume of 35 µl. These reactions were overlayed with 20 µl of mineral oil and incubated overnight at 60°C. All reactions were analysed by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD) and photographed using a polaroid land camera.

35 In all reactions which had not been subjected to digestion with *Bst* NI following the PCR, a 103 bp fragment generated by amplification with primers 5BKIM

and 3AKIL was visible. Following subsequent digestion with *Bst* NI, this 103 bp fragment was visible only in reactions containing Calu I:K562 DNA at ratios of 1:10, 1:10² and 1:10³. The 103 bp fragment was not visible in the reactions containing Calu I:K562 DNA at a ratio of 1:10⁴ nor in the reaction containing K562 DNA alone. A
5 73 bp fragment, generated by *Bst* NI digestion of the wild type amplicons, was visible in all reactions. In reactions which were digested with *Bst* NI following the PCR, the presence of the 103 bp fragment was diagnostic for the presence of a mutation at codon 12 of *K-ras*.

The sensitivity of this protocol allowed detection of mutant Calu I DNA when
10 present at a ratio of 1:10³ Calu I:K562 DNA. Under these reaction conditions, the inclusion of *Bst* NI in the PCR reaction resulted in preferential amplification (enrichment) of mutant sequences but did not result in complete inhibition of amplification of wild type K562 sequences. The reactions therefore required digestion with *Bst* NI prior to final analysis. Such protocols are of intermediate simplicity
15 between standard enriched PCR protocols (which require two rounds of PCR plus an intermediate digestion with a restriction endonuclease to enrich for mutant sequences) and standard REMS-PCR protocols (where amplification of wild type sequences is completely inhibited and no subsequent manipulations such as digestion are required prior to analysis).

20

DISCUSSION

In REMS-PCR protocols the restriction endonuclease and the DNA polymerase must i) function in identical reaction conditions (eg., salt, pH) which must be compatible with the PCR and ii) be sufficiently thermostable in these reaction
25 conditions to retain activity during the thermocycling which is required for the PCR. Some of the restriction endonucleases listed in Table 1, as well as other thermophilic restriction endonucleases, would be suitable for incorporation in REMS-PCR protocols provided buffer conditions can be identified which i) are compatible with restriction endonuclease activity and which maintain endonuclease activity while reactions are
30 thermocycling during PCR and ii) are compatible with simultaneous DNA polymerase activity and which maintain polymerase activity while thermocycling during the PCR.

As little was previously known about the ability of restriction endonucleases to retain activity during the thermocycling required for the PCR, an assay which is simple and easy to conduct was developed to identify candidate thermophilic restriction
35 endonucleases and reaction conditions. In the activity/thermostability assay, enzymatic activity of a restriction endonuclease, in a variety of reaction conditions, can be

compared following a defined number of thermocycles. In this assay, reactions are prepared which contain primers, dNTPs, and DNA polymerase in concentrations which are standard for the PCR. The reactions contain no template DNA but include the buffer system, with or without additional reagents, and the restriction endonuclease to be examined. The reactions are placed on a thermocycler, subjected to a high temperature and then thermocycled. After a defined number of thermocycles reactions are removed, plasmid DNA is added to the tubes and the reactions are incubated at the optimal temperature for the restriction endonuclease as specified by the manufacturer. The enzymatic activity of the restriction endonuclease can be assessed by the degree of cleavage of the plasmid DNA as visualized by gel electrophoresis.

The activity/thermostability assay identified various restriction endonucleases, including *Bst* NI, *Bsl* I, *Tru* 91 and *Tsp* 509 I, which are sufficiently thermostable under certain buffer conditions to retain moderate or full catalytic activity following the thermocycling which is essential for the PCR. The reaction conditions which were most effective at preserving catalytic activity during thermocycling were identified. The catalytic activity of restriction endonucleases following thermocycling varied depending on the pH and ionic strength of the buffer, the choice and concentration of monovalent cation (K^+ or Na^+), the concentration of free Mg^{2+} , and the presence of other additives including dithiothreitol (DTT). The influence of each of these components can depend on the other components in the buffer.

It is also likely that the enzymatic activity of restriction endonucleases could be preserved by reducing the temperatures and times for DNA denaturation during the PCR. Factors known to influence the melting temperature of duplex DNA molecules include salt concentration, and the presence of reagents such formamide, dimethyl sulfoxide, glycerol and ethylene glycol. These reagents are compatible with at least some PCR systems. Inclusion of these, or other reagents which affect the DNA melting temperatures, may allow the PCR to be performed at decreased denaturation temperatures and/or times. These reagents may also have a direct positive or negative influence on the activity and/or thermostability of the restriction endonucleases (and/or DNA polymerase). The influence on the activity of restriction endonucleases of various thermocycling profiles, in the presence of additional reagents, can be assessed by the thermostability/activity assay described above. Identification of additional thermophilic restriction endonucleases, and reaction conditions which preserve the activity of restriction endonucleases during thermocycling, can be achieved following routine testing using the activity/thermostability without the exercise of inventive skill.

For REMS-PCR, the reaction conditions must not only preserve catalytic activity of the restriction endonucleases but they must also be suitable for the PCR. The buffer conditions must therefore be compatible with activity and thermostability of a DNA polymerase during thermocycling. There are many commercially available DNA polymerases which can be used for the PCR. These vary widely in their general properties, including both their optimal buffer conditions and the range of conditions they can tolerate. Examination of efficiencies of various DNA polymerases in the PCR, under reaction conditions which are known to preserve restriction endonucleases activity, allows identification of compatible DNA polymerase/restriction endonuclease/buffer combinations. A range of reaction conditions which had been demonstrated to maintain activity of restriction endonucleases, were also assessed for their compatibility with the PCR using various sets of primers and various DNA polymerases. The influence of different components of the reaction conditions on the PCR varied for different primer pairs and can depend on the other reaction components. For this reason, specific primers sets which are required for a PCR should be tested in this manner. Conditions for a PCR which are compatible with the concurrent activity of a restriction endonuclease and a DNA polymerase, and which result in efficient amplification with specific primer pairs can be identified following routine testing without the exercise of inventive skill.

REMS-PCR requires that the recognition/cleavage site for the thermophilic restriction endonuclease spans the nucleotide(s) which are to be analysed for genetic variations. This site can either occur naturally or may be induced by primers which contain internal mismatches to the template. When recognition/cleavage sites for restriction endonucleases are induced by primers, the sites lie partially within the primer and partially within the synthesized sequence which lies 3' to the primer in the amplicons. Primers must therefore include any mismatched bases which are required for induction of the restriction endonuclease site, but must not overlap the bases which are to be analysed. Rules for designing PCR primers which contain mismatched bases near the 3' terminus have been established (S. Kwok, et al. 1990. Nucleic Acids Research 18, 999-10005). While some terminally mismatched primers amplify inefficiently and reduce the yield of specific amplicons by up to 100 fold, the majority will amplify as efficiently as fully matched primers. For example when the terminal 3' base in a primer is G it will extend on templates containing C, T or G, but not A, at the complementary position.

Recognition/cleavage sites can be more easily induced when the restriction endonuclease requires only a short tetranucleotide sequence for recognition (eg *T_{ru}* 91

or *Tsp* 509 I) or when they recognise multiple sequences (eg *Bst* NI).

Recognition/cleavage sites for restriction endonucleases which recognise short sequences which are interrupted are particularly amenable to induction. For example, *Bst* I recognises the sequences CCNNNNNNNGG, where N is any nucleotide. *Bst* I could be used to analyse mutations which occur at codons which encode either glycine (GGN) or proline (CCN). In general, primers designed to induce a *Bst* I recognition site at these codons could be extended by DNA polymerases since they would not require mismatched bases near the 3' terminus and single or double mismatches located in the middle of a primer sequence are well tolerated and do not usually inhibit PCR amplification.

Furthermore, one skilled in the art could design primers capable of inducing a *Bst* I recognition site for analysis of the vast majority (approximately 80%) of mutations that occur at either a G or a C. Mutation of the bases G and C are very common. For example, the percentage of p53 mutations that occur at either G or C residues is at least 77% of mutations in colorectal tumours, 72% of mutations in lung tumours, 74% of mutations in bladder tumours, 61% of mutations in breast tumours and 66% of mutations in brain tumours (M. Hollstein et al 1996 Nucleic Acids Research 24, 141-146). The following table lists all possible combination of sequences surrounding the bases C or G and the terminal bases which would be required for primers to induce CC or GG at these positions as part of the *Bst* I site. The template/primer combinations which are predicted to be compatible with PCR are indicated in Table 15.

Table 15

| Sequence of the template adjacent to the target base (underlined) N=A,C,G,T X=A,C,T Y=A,G,T | Primer Type Sense (5' primer) Anti-sense (3' primer) | Primer 3' base | Template 3' base | Compatible with PCR |
|--|--|----------------------------------|----------------------------------|---------------------|
| <u>GG</u> N | Sense | G | C | Yes |
| <u>AG</u> N | Sense | G | T | Yes |
| <u>CG</u> N | Sense | G | G | Yes |
| <u>TG</u> X | Sense | G | A | No |
| <u>NG</u> G | Sense | N = as per template (sense) | N = as per template (anti-sense) | Yes |
| <u>NCC</u> | Anti-sense | G | C | Yes |
| <u>NCT</u> | Anti-sense | G | T | Yes |
| <u>NCG</u> | Anti-sense | G | G | Yes |
| <u>YCA</u> | Anti-sense | G | A | No |
| <u>CC</u> N | Anti-sense | N = as per template (anti-sense) | N = as per template (sense) | Yes |

- Examples of either natural or inducible recognition/cleavage sites for thermophilic restriction endonucleases in genes associated with acquired diseases are listed in Table 16. In these examples, restriction endonucleases which recognize wild type sequences are identified. The list includes restriction endonucleases which are known to be compatible with REMS-PCR and other endonucleases which are potentially compatible with the method. Primers for analysis of these mutations must include the bases which require induction (indicated in bold) but must not overlap the bases which are to be analysed (underlined). *Ras* proto-oncogenes (*K-ras*, *H-ras* and *N-ras*) are frequently activated in wide variety of human cancers by the acquisition of point mutations at codons 12, 13 and 61. Since codons 12 and 13 of all three *ras* genes code for glycine, *Bst*I could be used for the analysis of the vast majority of *ras* mutations. A novel point mutation within intron D of *H-ras* has also been found in bladder carcinomas. Resistance of HIV strains to certain drugs is associated with the acquisition of point mutations.

Table 16

| Gene | Disease | Cause | Wild Type Sequence (Bases to be analysed) Restriction endonuclease recognition site and name (Bases requiring induction) |
|-------------------------|-------------------|---|---|
| K-ras N-ras H-ras | Cancer | Point mutations codons 12 and 13 eg K-ras codon 12 eg K-ras codon 13 | GTTGGAGCT <u>GG</u> CCNNNNNNN <u>GG</u> <i>Bst</i> I GGAGCTGGT <u>GG</u> CCNNNNNNN <u>GG</u> <i>Bst</i> I |
| K-ras | Cancer | Point mutations codon 12 | GCT <u>GG</u> CCT <u>GG</u> <i>Bst</i> NI |
| K-ras N-ras H-ras | Cancer | Point mutations codon 61-position 1 eg. H-ras | CCAGGAGGAGT CCNNNNNNN <u>GG</u> <i>Bst</i> I |
| H-Ras | Cancer | Point mutations codon 61-position 3 | CGCCGCCAGG CCNNNNNNN <u>GG</u> <i>Bst</i> I |
| H-ras | Cancer | Point mutations codon 61 (except A to T at position 2) | CCAGG CCAGG (CCTGG) <i>Bst</i> NI |
| H-ras | Bladder Cancer | Point mutations Intron D | GTAA TTAA <i>Tru</i> 9I |
| HIV-I | AZT resistance | Point mutations 1. codon 41 2. codon 70 3. codon 215 | 1. GAA <u>ATG</u> A <u>ATT</u> <i>Tsp</i> 509I GCA <u>ATG</u> <i>Bsr</i> DI 2. AA <u>ATGG</u> A <u>ATT</u> <i>Tsp</i> 509I 3. TTTACC TTAA <i>Tru</i> 9I |
| | ddI resistance | Point mutation codon 74 | AAAATTA AATT <i>Tsp</i> 509I |

A selection of genes which can harbour inheritable mutations associated with disease are listed in Table 17. The sequences listed are either wild type or mutant and the positions of potential sequence variations are underlined. Analysis of recessive mutations requires discrimination between heterozygous carriers and homozygous individuals with the latter at risk of disease development. For all of the following examples, restriction endonuclease which would recognize the wild type sequences are identified. For the cystic fibrosis transmembrane conductance gene, restriction endonucleases which recognize the mutated sequence have also been identified

Table 17

| Gene | Disease | Sequence to be analysed | Type of Sequence/Sequence (Bases to be analysed) Endonuclease sites and names (Bases requiring induction) |
|--|------------------------------|--|--|
| Cystic fibrosis trans-membrane conductance regulator | Cystic fibrosis | Point mutations at | Wild type sequence |
| | | 1. codon 542 | 1. ATAGTTCTTGG CCNNNNNNNNGG <i>Bsl</i> I CCTGG <i>Bst</i> NI |
| | | 2. codon 551 | 2. CTGAGTGGAGGTCA CCNNNNNNNNGG <i>Bsl</i> I GGTCC <i>Bsi</i> ZI |
| | | 3. IVS-4 | 3. TTATAAGAAGG CCNNNNNNNNGG <i>Bsl</i> I |
| | | 4. Deletion codon 508 (3bp) | 4. AAATATCATCTT GATNNNNATC <i>Bsa</i> BI <i>Bsi</i> BI |
| | | Wild type sequences | Mutant sequence |
| | | 1. codon 542 | 1. TCTTTGA TTAA <i>Tru</i> 9I |
| | | 2. codon 551 | 2. GATCAACGAG GATNNNNATC <i>Bsa</i> BI <i>Bsi</i> BI |
| α -antitrypsin | Emphysema Liver cirrhosis | point mutation codon 342 | Wild type sequence GACCATCGACG CCNNNNNNNNGG <i>Bsl</i> I |
| | | Point mutation IVS-1 (β^0 -Mediterranean) | Wild type sequence CCCTGGGCAGG CCNNNNNNNNGG <i>Bsl</i> I |
| β -globin | β -Thalassemia | Point mutation poly A signal (β^+ -Black) | Wild type sequence AATAAA TTAA <i>Tru</i> 9I |

Little was previously known about the effect of including a thermostable restriction endonuclease in a PCR. It was discovered that simultaneous activity of a restriction endonuclease and a DNA polymerase during the PCR can result in (i) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) selective amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. This discovery allows the development of protocols known as REMS-PCR. Such protocols could be used for the analysis of acquired or inherited polymorphisms, including point mutations, small deletions and insertions. When protocols for REMS-PCR are designed to detect mutant sequences, the wild type but not mutant sequences contain the recognition/cleavage sequence for a thermophilic restriction endonuclease. Amplification of wild type sequences by the PCR is inhibited by the activity of the restriction endonuclease. In contrast, mutant sequences are selectively amplified by DNA polymerase during the PCR.

Protocols for REMS-PCR can also be designed to selectively inhibit amplification of mutant but not wild type sequences. If protocols for REMS-PCR are designed to detect wild type sequences, the mutant but not wild type sequences contain the recognition/cleavage sequence for a thermophilic restriction endonuclease. Amplification of mutant sequences by the PCR would be inhibited by the activity of the restriction endonuclease and wild type sequences would be selectively amplified by the PCR. Failure to amplify specific wild type sequences would be consistent with a homozygous mutation. The ability to detect both wild type and mutant sequences would be consistent with the presence of a heterozygous mutation.

Several protocols for REMS-PCR were developed for the analysis of point mutations at codon 12 of the *K-ras* oncogene. These protocols exploited concurrent enzymatic activity of *Bst* NI and DNA *Taq* polymerase, or *Bst* NI and Stoffel fragment polymerase, or *Bst* I and DNA *Taq* polymerase. These protocols include multiplex primer systems which comprise diagnostic primers and one or two sets of control primers. The diagnostic primers induce a recognition/cleavage site for either *Bst* NI or *Bst* I in *K-ras* amplicons provided positions 1 and 2 of codon 12 are wild type. Inclusion of one of these restriction endonucleases in the PCR results in inhibition of amplification of wild type DNA templates and selective amplification of DNA templates which contain mutations at positions 1 or 2 of codon 12. Amplification with these primers is therefore diagnostic for the presence of a point mutation at codon 12. Additional control primers are included in all reactions to confirm that the reaction conditions, including the amount of template DNA, are adequate for amplification by

the PCR. These PCR control primers can flank any region which does not contain the endonuclease recognition/cleavage site. Amplicons incorporating these primers must be present for unambiguous interpretation of negative results. A second control primer was included in one multiplex system to confirm that the restriction endonuclease could
5 mediate complete inhibition of amplification by the PCR. Control primers for the restriction endonucleases must either induce or flank the recognition/cleavage site for the restriction endonuclease used in the REMS-PCR protocol. Absence of amplicons incorporating these primers allows unambiguous interpretation of positive results.

The limits of detection of the REMS-PCR were assessed by analysis of
10 samples containing Calu I DNA (heterozygous mutant at *K-ras* codon 12) diluted in Sup T1 DNA (wild type at *K-ras* codon 12) in the presence of *Bst* NI and DNA *Taq* polymerase. The detection of diagnostic amplicons indicated the presence of *K-ras* sequences which were mutated at codon 12. Diagnostic amplicons were visualized, using gel electrophoresis and colorimetric analysis, in samples containing Calu I:Sup T1
15 at ratios of 1:10 to 1:10,000 but not in samples containing Sup T1 alone. PCR control amplicons were detected in all samples including Sup T1 DNA. The literature suggests that this level of sensitivity will be adequate for analysis of DNA extracted from clinical specimens including tissue resections and biopsies, cytology samples and body fluids/excretions such as stools, urine and sputum containing small numbers of exfoliate
20 tumour cells (D.Sidransky et al. ,1992 Science 256, 102-1; L.Mao et al. 1994 Cancer Res. 54, 1634-1637). The application of REMS-PCR to the analysis of clinical specimens was demonstrated. Mutations at *K-ras* codon 12 were detected in DNA extracted from two out of four colon adenocarcinomas but none were detected in DNA extracted from four normal colon mucosas.

25 In an extension of the REMS-PCR, the protocol can be performed with primers which simultaneously induce i) a recognition/cleavage site for a restriction endonuclease that is present only in the wild type sequence and ii) multiple recognition/cleavage sites for restriction endonucleases that are specific for all possible mutated sequences. Subsequent analysis of diagnostic amplicons with the restriction
30 endonucleases allows confirmation of the presence of a mutation in these amplicons and allows identification of the exact nucleotide substitutions in all cases.

It is also possible to develop REMS-PCR systems which result in selective amplification of mutant sequences but which do not result in complete inhibition of amplification of wild type sequences or *vice versa*. Reactions therefore require
35 digestion with appropriate restriction endonuclease following PCR prior to analysis. Such protocols are of intermediate simplicity between standard enriched PCR protocols

and standard REMS-PCR protocols where amplification of wild type sequences is completely inhibited.

REMS-PCR is compatible with a variety of capture and detection systems. This allows automation of the complete protocol and thus rapid analysis of large numbers of samples. Examples of capture systems include but are not restricted to

5 i) PCR primers with a GCN4 recognition tag captured on GCN4 coated plates; ii) biotinylated primers captured with avidin or streptavidin; iii) digoxigenin-labelled products captured using anti-digoxigenin antibodies; and iv) complementary oligonucleotides attached to latex or magnetic beads. Examples of detection systems

10 include, but are not restricted to, i) biotinylated PCR primers visualized with streptavidin/ horse radish peroxidase; ii) direct labelling with molecules such as fluorescein-isothiocyanate or alkaline phosphatase; and iii) digoxigenin-labelled products detected using anti-digoxigenin antibodies.

REMS-PCR provides a sensitive, rapid method which is suitable for analysis of

15 genetic variations which are associated with disease. The ability to simultaneously sustain the activities of a restriction endonuclease and a DNA polymerase during the PCR allows the development of simple protocols for selective amplification of variant sequences in reactions which contain all reagents, including all enzymes, at the initiation of the PCR. Reactions can be performed in a closed system which reduces the

20 opportunity for contamination during the PCR. The protocol for REMS-PCR has fewer steps than other protocols which utilize restriction endonucleases to mediate selective amplification and/or analysis of mutant sequences. In general, the reactions do not require further manipulation prior to detection, however, the method does not preclude subsequent analysis of diagnostic amplicons for identification of the exact

25 nucleotide substitution. A reduction in the number of steps required for selective amplification and analysis with restriction endonucleases makes the REMS-PCR assay rapid, less labour intensive and more amenable to automation.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific

30 embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References

- 5 Capon, D.J., Seeburg, P.H., McGarth, J.P., Hayflick, J.S., Edmund, U.,
Levinson, A.D. and Goeddel, D.V. (1983) Activation of Ki-ras 2 gene in human colon
and lung carcinomas by two different point mutations. *Nature* 304, 507-513.
- 10 Chehab, F.F., Doherty, M., Cai, S., Kan, Y.W., Copper, S. and Rubin, E.M.
(1987) Detection of sickle cell anaemia and thalassaemias. *Nature* 329, 293-294
- 15 Cohen, J.B. and Levinson A.D. (1988) A point mutation in the last intron
responsible for increased expression and transforming activity of the c-Ha-ras
oncogene. *Nature* 334, 119-124.
- 20 Kumar R. and Barbacid M. (1988) Oncogene detection at the single cell level.
Oncogene 3, 647-651
- 25 Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C.,
and Sninsky, J.J. (1990) Effect of primer-template mismatches on the polymerase chain
reaction: Human immunodeficiency virus type I model studies. *Nucleic Acids
Research.*, 18, 999-10005).
- 30 Levi, S., Urbano-Ispizua, A., Gill, R., Thomas D.M., Gilbertson J., Foster C.
and Marshall C.J. (1991) Multiple K-ras codon 12 mutations in cholangiocarcinomas
demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res.* 51,
3497-3502
- 35 Mao, L., Hruban R.H., Boyle J.O., Tockman M. and Sidransky D. (1994)
Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer
Res.* 54, 1634-1637.
- 40 Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and
Arnheim, N. (1985) Enzymatic amplification of β -globin genomic sequences and
restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn, New York: Cold Spring Harbour Laboratory Press.

- 5 Sharkey, D.J., Scalice, E.R., Christy, K.G.Jr., Atwood, S.M. and Daiss, J.L. (1994) Antibodies as thermolabile switches: High temperature triggering for the polymerase chain reaction. *Bio/technology* 12, 506-509.

- 10 Sidransky, D., Tokino T., Hamilton S.R., Kinzler K.W., Levin B., Frost P. and Vogelstein B. (1992) Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 256, 102-105.

- 15 Singh, J., Rao, C.V., Kulkarni N., Simi B. and Reddy B.S. (1994) Molecular markers as intermediate end-points in chemoprevention of colon cancer: Modulation of ras activation by sulindac and phenylhexylisothiocyanate during colon carcinogenesis. *Int.J.Oncol.* 5, 1009-1018.

- 20 Todd, A.V., Ireland, C.M. and Iland, H.J. (1991) Allele-specific enrichment: A method for detection of low level N-ras gene mutations in acute myeloid leukemia. *Leukemia* 5, 160-161.

Valenzuela, D.M. and Groffen J. (1986) Four human carcinoma cell lines with novel mutations in position 12 of the c-K-ras oncogene. *Nucleic Acids Research* 14, 843-852

- 25 Ward, R.L., Santiago, F., Hawkins, N.J., Coomber, D., O'Connor, T. and Todd, A.V. (1995) A rapid PCR ELISA for the detection of activated K-ras in colorectal cancer. *J Clin Pathology: Mol Pathol.* 48, M273-277.

Patents cited

30

WO 84/01389 (Weinberg et al. Massachusetts Institute of Technology)

EPO 684 315 AI (Becton Dickinson and Company)

- 35 US 4683202 (Mullis, K.B. Cetus Corporation)

US 4683195 (Arnheim, N. et al. Cetus Corporation)

US 4800159 (Arnheim, N. et al. Cetus Corporation)

5 US 4965188 (Ehrlich H.A. et al. Cetus Corporation)

US 5176995 (Erlich H.A. et al. Hoffmann-La Roche Inc.)

CLAIMS:

1. A method of detecting a genetic polymorphism in an individual, the method comprising the following steps:
 - 5 (1) Obtaining a sample containing nucleic acid from the individual;
 - (2) Amplifying the nucleic acid sample from step (1) by a process involving thermocycling and primers, the amplification occurring in the presence of a thermostable restriction endonuclease which retains activity during thermocycling, the primers being selected such that they introduce into either
10 the nucleic acid amplified from nucleic acid not including the polymorphism or from nucleic acid including the polymorphism, a sequence recognised by the thermostable restriction endonuclease; and
 - (3) Analysing the product of step (2) to determine the presence or absence of the polymorphism.
- 15 2. A method as claimed in claim 1 in which the primers introduce the sequence recognised by the thermostable restriction endonuclease into the nucleic acid amplified from the nucleic acid not including the polymorphism.
3. A method of detecting a genetic polymorphism in an individual, the method comprising the following steps:
 - 20 (1) Obtaining a sample containing nucleic acid from the individual;
 - (2) Amplifying the nucleic acid sample from step (1) by a process involving thermocycling and primers, the amplification occurring in the presence of a thermostable restriction endonuclease having concurrent activity, the restriction endonuclease being selected such that it recognises nucleic acid not
25 including the polymorphism but not nucleic acid including the polymorphism or *vice versa*; and
 - (3) Analysing the product of step (2) to determine the presence or absence of the polymorphism.
4. A method as claimed in claim 3 in which the thermostable restriction
30 endonuclease recognises nucleic acid not including the polymorphism.
5. A method as claimed in claim 2 or claim 4 in which the method further comprises the following additional steps of:
 - (4) reacting the amplified nucleic acid from step (2) with at least one restriction endonuclease, the at least one restriction endonuclease being
35 selected such that it digests the amplified nucleic acid including a particular polymorphism; and

(5) determining whether digestion occurs in step (4), digestion being indicative of the presence of the particular polymorphism.

6. A method as claimed in any one of claims 1 to 5 in which the process involving thermocycling is PCR.
- 5 7. A method as claimed in any one of claims 1 to 6 in which the step (3) analysis comprises detecting the presence or absence of amplified nucleic acid from step (2), the presence or absence of amplified nucleic acid indicating the presence or absence of the polymorphism.
8. A method as claimed in any one of claims 1 to 7 in which the nucleic acid is
10 DNA.
9. A method as claimed in any one of claims 1 to 8 in which the thermostable restriction endonuclease is selected from the group consisting of *Bst* NI, *Bsl* I, *Tru* 9I and *Tsp* 509 I.
10. A method as claimed in any one of claims 1 to 9 in which the genetic
15 polymorphism is detected in one of the *ras* proto-oncogenes, *K-ras*, *N-ras*, and *H-ras*, or the p53 tumour suppressor gene.
11. A method as claimed in claim 10 in which the genetic polymorphism is detected in codon 12 of *K-ras*.
12. A method as claimed in any one of claims 1 to 9 in which the genetic
20 polymorphism is detected in HIV-I, cystic fibrosis trans-membrane conductance regulator, α -antitrypsin or β -globin.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00213

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : as above : FILE WPAT (see below)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above : FILE WPAT (see below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
FILE WPAT: Keywords: polymerase chain reaction, PCR, ligase chain reaction, LCR, amplify, amplification, amplifies, restriction endonuclease(s)
FILE CASM (Chemical Abstracts) : Keywords: as above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | Singh, J. et al. International Journal of Oncology, Volume 5 (1994) "Molecular markers as intermediate end-points in chemoprevention of colon cancer: Modulation of <u>ras</u> activation by sulindac acid and phenylhexylisothiocyanate during colon carcinogenesis" 1009 to 1018. See page 1011, column 2, lines 16 to 42. | 1 to 12 |
| Y | Walker, G.T. et al. Proceedings of the National Academy of Sciences, USA, Volume 89 (January 1992) "Isothermal <u>in vitro</u> amplification of DNA by a restriction enzyme/DNA polymerase system" 392 to 396 - See whole document | 1 to 12 |



Further documents are listed in the continuation of Box C



See patent family annex

| | |
|---|--|
| <p>* Special categories of cited documents:</p> | |
| "A" document defining the general state of the art which is not considered to be of particular relevance | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "E" earlier document but published on or after the international filing date | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" document referring to an oral disclosure, use, exhibition or other means | "&" document member of the same patent family |
| "P" document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search
28 May 1996

Date of mailing of the international search report

6TH JUNE 1996.

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
PO BOX 200
WODEN ACT 2606
AUSTRALIA Facsimile No.: (06) 285 3929

Authorized officer

MARK ROSS

Telephone No. (06) 283 2295

INTERNATIONAL SEARCH REPORTInternational Application No.
PCT/AU 96/00213

| C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|--|------------------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| P,Y | AU,A,14776/95 (BECTON, DICKINSON & COMPANY) 26 October 1995. See whole document. | 1 to 12 |
| Y | AU,B,10699/92 (659429) (BECTON, DICKINSON & COMPANY) 29 July 1993. See whole document | 1 to 12 |
| Y | AU,B,10264/92 (652214) (BECTON, DICKINSON & COMPANY) 6 August 1992. See whole document. | 1 to 12 |
| P,Y | EP,A,678582 (BECTON, DICKINSON & COMPANY) 25 October 1995. See whole document. | 1 to 12 |

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00213

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | | | Patent Family Member | | | |
|--|----------|----|----------|----------------------|----------|----|----------|
| AU | 14776/95 | BR | 9501581 | CA | 2144495 | EP | 684315 |
| | | JP | 7289298 | | | | |
| AU | 10699/92 | BR | 9300028 | CA | 2060372 | EP | 497272 |
| | | JP | 5192195 | US | 5455166 | AU | 10264/92 |
| | | CA | 2060371 | | | | |
| AU | 10264/92 | CA | 2060371 | CA | 2060372 | EP | 497272 |
| | | JP | 5192195 | US | 5455166 | AU | 10699/92 |
| | | BR | 9300028 | | | | |
| EP | 678582 | AU | 15023/95 | BR | 9501583 | CA | 2145576 |
| | | CA | 2145719 | EP | 678581 | JP | 7289299 |
| | | JP | 8038199 | AU | 15019/95 | | |
| END OF ANNEX | | | | | | | |